Sensitivity to NNKOAc is associated with renal cancer risk

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Cigarette smoking has been investigated as a major risk factor for renal cell carcinoma (RCC). 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most abundant carcinogenic *N*-nitrosamines present in cigarette smoke. However, the association between repair capacity of NNK-induced DNA damage and RCC risk remains unknown. We used the comet assay to assess whether sensitivity to a NNK precursor 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone (NNKOAc) induced DNA damage, which partly reflects host sensitivity to NNK, was associated with increased risk of RCC in a population-based case-control study. The study included 95 RCC cases and 188 matched controls. Epidemiologic data were collected via in-person interview. Baseline and NNK-induced DNA damage in peripheral blood lymphocytes were measured using the comet assay and quantified by the Olive tail moment. The NNKOAc-induced median Olive tail moments were significantly higher in cases than in controls (2.27 versus 1.76, $P = 0.002$). Using the 75th percentile Olive tail moments of the controls as the cutoff point, we found that higher levels of NNKOAc-induced DNA damage were associated with a significantly increased risk of RCC [odds ratio, 2.06; 95% confidence interval, 1.17–3.61]. In quartile analysis, there was a dose–response association between NNKOAc-induced damage and risk of RCC (P for trend, 0.006). Our data strongly suggest that higher levels of NNKOAc-induced damage are associated with higher risks of RCC. Future studies with larger sample sizes are warranted to further investigate whether repair of NNKOAc-induced damage, as quantified by the comet assay, could be used as a predictive marker for RCC risk.

Introduction

Cigarette smoking has been investigated as a major risk factor for renal cell carcinoma (RCC), but the results remain inconsistent (1,2). According to a recent meta-analysis conducted by Hunt *et al.* (2), ever smokers were at a 1.38-fold increased risk of RCC compared with lifetime never smokers [95% confidence interval (CI), 1.27–1.50]; however, of the 24 studies examined, 12 rendered non-significant results. Likewise, a systematic review by Dhote *et al.* (3) with 11 studies examining risk factors for renal cell cancer found an association between smoking and RCC in only seven. Furthermore, when examining RCC studies whose primary exposure of interest was not smoking, we have observed non-significant associations between smoking and RCC; for example, a family history of kidney cancer study performed in Central Europe by Hung *et al.* (4) reported 47% of cases being ever smokers compared with 40% of controls and 30% of cases being current smokers compared with 35% of controls. Simi-

Abbreviations: BMI, body mass index; CI, confidence interval; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNKOAc, 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone; OR, odds ratio; RCC, renal cell carcinoma; XP, xeroderma pigmentosum.

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larly, in the current study, we did not observe a significant association between cigarette smoking and RCC risk and we believe that inter-individual differences in genetic susceptibility to tobacco-induced DNA damage may be contributing to this inconsistency.

Cigarette smoke contains a variety of carcinogenic chemicals such as polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic aromatic amines and *N*-nitrosamines. Among the *N*-nitrosamines present in cigarette smoke, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most abundant and the most potent in terms of carcinogenicity (5–7). NNK requires α -hydroxylation for metabolic activation, producing two types of DNA damage—methylation and pyridyloxobutyl adducts—that are involved in the carcinogenesis of NNK-related cancers (8). DNA damage requires a DNA repair system that will recognize and repair the damage through complex pathways (9). Failure to repair the DNA damage may lead to genomic instability and subsequent carcinogenesis (10). The mechanism to repair NNK is not clear, but nucleotide excision repair is a major pathway to remove DNA adducts and may contribute to inherited susceptibility to cancer (11,12). In a previous study, we showed that sensitivity to benzo[*a*]pyrene diol epoxide was associated with increased risk for RCC (13). However, the association between repair capacity of NNK-induced DNA damage and RCC risk has yet to be explored.

DNA damage/repair capacity can be quantified by phenotypic assays such as the mutagen sensitivity assay, host cell reaction and comet assay (14). Comet assay, also known as single cell gel electrophoresis (SCGE) technique, is a well established, technically simple, relatively fast and inexpensive functional assay to measure DNA damage at the individual cell level (15,16). However, the study of DNA damage by NNK is difficult due to its complex enzymatic activation (8). To bypass this problem, a precursor 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone (NNKOAc) has been used in previous reports. NNKOAc, which pyridyloxobutylates DNA, induces single-strand breaks in a concentration-dependent manner. NNKOAc generates damage at all four bases with decreasing order; guanine > adenine > cytosine > thymine (17). The comet assay has been shown to be an effective way to measure NNKOAc-induced damage levels (18–20). Furthermore, xeroderma pigmentosum (XP) cell lines are more susceptible to NNKOAc-induced cytotoxicity (12). Therefore, in the current study, we used the comet assay to assess whether sensitivity to NNKOAc-induced DNA damage was associated with increased risk of RCC in a population-based case–control study among 95 RCC cases and 188 matched controls.

Materials and methods

Study population

Beginning in 2002, incident RCC cases were recruited from The University of Texas M. D. Anderson Cancer Center in Houston, TX. All cases were individuals with newly diagnosed, histologically confirmed RCC and were residents of Texas. There were no age, gender, ethnicity or cancer stage restrictions on recruitment. M. D. Anderson staff interviewers identified RCC cases through a daily review of computerized appointment schedules for the Departments of Urology and Genitourinary Medical Oncology. Healthy control subjects without a history of cancer, except non-melanoma skin cancer, were identified and recruited using the random digit dialing methods. In random digit dialing, randomly selected phone numbers from households were used to contact potential control volunteers in the same residency of cases according to the telephone directory listings. Controls must have lived in the same county or socioeconomically matched surrounding counties that the case resides in for at least 1 year and have had no prior history of cancer. The controls were frequency matched to the cases by age (± 5 years), sex, ethnicity and county of residence. The response rate among cases was 87%. The initial random digit dialing screening response rate among controls was 51% and among eligible controls the response rate was 87%.

Epidemiologic data

After written informed consent was obtained, all study participants completed a 45 min in-person interview that was administered by M. D. Anderson Cancer

Center staff interviewers. The interview elicited information on demographics, smoking history, family history of cancer, occupational history and exposures and medical history. At the conclusion of the interview, a 40 ml blood sample was drawn into coded heparinized tubes and delivered to our laboratory for molecular analysis. The study was approved by the Institutional Review Board of M. D. Anderson Cancer Center. An individual who had smoked at least 100 cigarettes in his or her lifetime was defined as an ever smoker. Ever smokers included former smokers (those who had quit smoking for at least 1 year), current smokers and recent quitters (those who had quit within the previous year). Body mass index (BMI, kg/m²) was calculated using self-reported height and usual weight. High blood pressure was assessed by whether a participant answered yes or no to ever having been told by a doctor if they had hypertension or high blood pressure.

NNKOAc comet assay

NNKOAc was obtained from Toronto Research Chemicals (North York, Ontario, Canada) and 3.77 ml of dimethyl sulfoxide were added to vials containing 10 mg NNKOAc (molecular weight 265.27) to make a 10 mM working solution. The vials were then filled with argon and stored at -80°C following the manufacturer's instructions.

The duration of NNKOAc treatment had been optimized to be 24 h based on data from mutagen sensitivity assays (data not shown). The optimal dosage of NNKOAc was determined first in Epstein-Barr virus-transformed lymphoblastoid cell lines and then in blood samples. Four types of lymphoblastoid cell lines were used in this study: GM 2248 (XP-C deficient), GM 2253 (XP-D deficient), GM 2345 (XP-A deficient), GM 2450 (XP-5 deficient) from patients with XP (Coriell Institute of Medical Research, Camden, NJ), LW 510 from a normal blood donor and LSE 84 and LSE 94 from patients with lung adenocarcinoma (gifted by University of Texas Southwestern Medical Center). These cell lines were grown with 50 U/ml penicillin G sodium and 50 µg/ml streptomycin sulfate in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 15% fetal bovine serum (Sigma-Aldrich Corp., St Louis, MO). For use in the comet assay, cell cultures were in the logarithmic growth phase with a concentration of 0.5×10^6 cells/ml. The comet assay was performed after 24 h continuous treatment of NNKOAc with doses of 0, 25, 50, 75, 80, 90, 100 and 110 µM.

The optimal dosage for blood samples in the comet assay was decided by comparing the data in random lung cancer samples and controls. Blood cultures were set up immediately after the samples were delivered to the laboratory. A total of 0.4 ml of whole blood was cultured in 1.6 ml of RPMI 1640, with 15% fetal calf serum and 1.25% phytohemagglutinin (Wellcome Research Laboratories, Research Triangle Park, NC), in 60 × 15 mm Petri dishes (Falcon, Franklin, NJ) at 37°C for 96 h. Separate blood cultures were prepared for baseline and NNKOAc-induced comet assay analyses. At the 72h hour, we added NNKOAc to the culture in diminished light and covered the Petri dish with foil to reduce the DNA damage caused by the light exposure. For the baseline culture, no NNKOAc was added to the culture. Multiple doses (0, 100, 200, 250, 300, 350, 400 and 500 µM) had been selected for the testing of NNKOAc comet assay and the dose of 250 µM was decided to be optimal.

We used a modified version of the alkaline comet assay method originally described by Singh *et al.* (16). All procedures were performed with reduced illumination to minimize UV exposure. Briefly, 50 µl of blood culture were mixed with 150 µl of 0.5% low-melting agarose (Life Technologies). A total of 50 µl of mixture was layered on each end of microscope slides that were precoated with 1% agarose. After the mixture solidified, a third layer of 0.5% low-melting agarose was placed on top of the cell culture/low-melting agarose layer on the same slide and covered with a coverslip. After the third layer of agarose was completely solidified, the cells were lysed for 1 h at 4°C in freshly prepared 1× lysis buffer [2.5 M of NaCl, 100 mM of ethylenediamine tetraacetic acid, 1% sodium sarcosinate, 10 mM Tris (pH 10), 10% dimethyl sulfoxide and 1% Triton). After cell lysis, the slides were treated with alkali buffer [300 mM of NaOH and 1 mM of ethylenediamine tetraacetic acid (pH > 13)] at 4°C in a horizontal electrophoresis box without power for DNA denaturing, unwinding and exposure of the alkali-labile site. Next, a constant electric current of 295–300 mA was applied for 23 min at 4°C to separate damaged DNA from the nuclei. After electrophoresis, the slides were neutralized by three 5 min washes in 0.4 M Tris-HCl (pH 7.4). The slides were then stained by ethidium bromide solution (10 mg/ml in 0.4 M Tris buffer) for 2 min and scored within 2 days.

Fifty cells of each sample (25 from each end of the slide) were randomly picked to reduce intra-assay variation and analyzed using Komet 5.5 imaging software (Kinetic Imaging Ltd, Liverpool, UK). The Olive tail moment was calculated as [(tail mean - head mean) × (tail %DNA/100)] and used as the parameter for measuring DNA damage (15). The mean Olive tail moment was obtained for the baseline comet and NNKOAc-induced comets for each study subject. All the comet assays included in this study were performed within

a period of one and a half years by one of the authors and each step of the comet assay was followed strictly. Standardized scanning criteria were followed. The laboratory personnel had no knowledge on case-control status of the samples.

Statistical analysis

The χ^2 test was used to test for differences between cases and controls for categorical data including the distribution of sex, ethnicity, smoking status, BMI and history of hypertension. For continuous variables such as age and pack-years, the Student's *t*-test or non-parametric Wilcoxon rank sum test was used when appropriate. Olive tail moments were analyzed as continuous variables using the Wilcoxon rank sum test. Baseline, NNK-induced and NNK-baseline Olive tail moments were dichotomized by 75th percentile or by quartiles based on the distribution of the control group. Unconditional multivariable logistic regression was performed to estimate odds ratios (ORs) while adjusting for age, sex, ethnicity, smoking status, BMI and history of hypertension, when appropriate. In order to adjust for the possibility of laboratory drift over the one and a half year study period, we performed a log transformation of the Olive tail moment variables and calculated their residuals based on a linear regression including time. We then repeated all the previously stated analyses. All statistical analyses were performed with the Stata 8.2 statistical software package (Stata Corporation, College Station, TX) and were two sided.

Results

Figure 1 shows the NNKOAc dose-response curve in three types of lymphoblastoid cell lines with treatment duration of 24 h. The net NNKOAc-induced DNA damage, reflected by Olive tail moments increased with the greater dosages of NNKOAc. At the lower dosages (25 and 50 µM), the DNA damage induced by NNKOAc was indistinguishable among the three types of cell lines. At the doses of 75 and 80 µM, the GM cell lines, which have constitutional deficiencies in nucleotide excision repair system, showed the greatest NNKOAc sensitivity, followed by LSE cell lines of patients with lung adenocarcinoma. The LW cell line, which was derived from a healthy control, had the lowest NNKOAc sensitivity. DNA damage reached a plateau at 75 µM for GM cell lines, whereas it increased in LSE and LW cell lines with an increase in NNKOAc dose.

To produce similar genotoxicity in our blood samples, final NNKOAc concentrations of 100, 200, 250, 300, 350, 400 and 500 µM were used to determine the optimal dose. The mean net NNKOAc-induced Olive tail moments for dosages of 100, 200, 250, 300, 350, 400 and 500 µM were

Fig. 1. NNKOAc dose-response curve in three types of lymphoblastoid cell lines and blood samples. The x-axis on the top represents the NNKOAc dose used in blood samples (cases and controls) and the x-axis on the bottom represents the dose used in cell lines (GM, LSE and LW cell lines). GM cell lines include four XP-deficient cell lines: GM 2248, GM 2253, GM 2480 and GM 2345; LSE cell lines include two cell lines from patients with lung adenocarcinoma: LSE 84 and 94; LW cell line is a cell line LW 510 from a healthy donor. Blood samples included five pairs of cases and controls. The data presented here were net NNKOAc-induced Olive tail moment (baseline data were subtracted from NNKOAc-induced data). Note the dose-dependent increase of DNA damage in each cell line group.

1.17 versus 0.82, 1.62 versus 1.21, 2.29 versus 0.99, 3.01 versus 1.80, 3.16 versus 1.91, 2.69 versus 2.93 and 2.70 versus 2.37 in five pairs of cases and controls, respectively (Figure 1). With the increasing NNKOAc dosage, especially at 350 μ M or higher, increased numbers of lymphocytic cells showed toxic apoptosis. At the dosages of 250 and 300 μ M, the net NNKOAc-induced Olive tail moment between cases and controls exhibited the greatest difference without cytotoxicity. We, therefore, chose the dosage of 250 μ M as the final treatment concentration.

Table I summarizes the host characteristics of the study population. As shown, by study design, the case patients were successfully matched to the controls by age, sex and ethnicity (all P -values > 0.05). Although several smoking variables, such as ever smoking, pack-years and years smoked, were higher in cases than controls, they were not statistically significant differences. Half of the cases had a BMI of at least 30 compared with 31.9% of controls. Similarly, only 17.9% of cases had a BMI of <25 compared with 29.8% of controls ($P = 0.007$). Among the cases, 56.8% reported history of hypertension as compared with 39.9% in controls ($P = 0.007$).

At baseline, the DNA damage reported as median Olive tail moments were not significantly different between cases and controls (Table II, 1.08 versus 1.02, $P = 0.112$). However, NNKOAc-induced

Olive tail moments were significantly higher in cases than in controls (2.27 versus 1.76, $P = 0.002$). When NNKOAc-induced damage were subtracted by baseline DNA damage, these differences remained significant (1.02 versus 0.78, $P = 0.018$).

We then analyzed the data stratified by smoking status (Table III). Among former smokers, cases showed significantly higher levels of NNKOAc-induced DNA damage as compared with controls (2.42 versus 1.96, $P = 0.023$). Similarly, when baseline damage was subtracted from NNKOAc-induced damage, the difference remained significant (1.19 versus 0.85, $P = 0.038$). No statistically significant differences were observed among current smokers. However, the trend of higher damage in cases than in controls was suggestive among never smokers with a borderline significant difference.

Next, we analyzed the association between DNA damage levels and risk for RCC (Table IV). Using the 75th percentile Olive tail moments of the controls as the cutoff point, we found that higher levels of NNKOAc-induced DNA damage were associated with a significantly increased risk of RCC (OR of 2.06, 95% CI, 1.17–3.61). In quartile analysis, there was a dose–response association between NNK-induced damage and risk of RCC. Compared with individuals within the lowest quartile of NNKOAc-induced damage (the first quartile), the ORs for the second, third and fourth quartiles were 1.61 (95% CI, 0.69–3.75), 1.77 (95% CI, 0.76–4.09) and 3.00 (95% CI, 1.36–6.62) with a significant trend (P for trend, 0.006). Similar findings were obtained when baseline DNA damage was subtracted from NNKOAc-induced DNA damage. The association between DNA damage at baseline and RCC risk was not significant in either the 75% percentile or in quartile analyses.

We stratified the analysis by smoking status and estimated the risk of RCC based on DNA damage repair (data not shown). Using the 75th percentile Olive tail moments of the controls as the cutoff point, we found that high levels of NNKOAc-induced damage were associated with a >2 -fold increased risk of RCC among former smokers and never smokers (OR of 2.52, 95% CI, 0.97–6.54 for former smokers and OR of 2.29, 95% CI, 1.02–5.13 for never smokers). When we subtracted baseline damage from NNKOAc-induced damage, a similar association was shown only among never smokers (OR of 2.14, 95% CI, 0.93–4.93). The small number of current smokers prohibited meaningful assessment among that group.

Discussion

Cigarette smoking has been investigated as a major risk factor for RCC. A major component of cigarette smoke, NNK, is a known carcinogen and inducer of DNA damage. However, the association between the repair capacity of NNK-induced DNA damage and RCC risk remains unknown. Therefore, in this case–control study, we used the comet assay to measure baseline and NNKOAc-induced DNA damage and analyzed the association between the level of DNA damage and risk of RCC.

Significant associations were observed between Olive tail moments and risk of RCC. Significantly higher NNKOAc-induced median Olive tail moments were observed in cases than in controls. Using the 75th percentile Olive tail moments of the controls as the cutoff point, we found that higher levels of NNKOAc-induced DNA damage were

Table I. Distribution of selected host characteristics by case–control status

Variables	Case patients ($n = 95$)	Control subjects ($n = 188$)	P -value ^a
Sex, no. (%) ^b			
Male	70 (73.7)	127 (67.6)	0.290
Female	25 (26.3)	61 (32.5)	
Age, mean $\pm$ SD	57.3 $\pm$ 9.6	59.8 $\pm$ 11.2	0.064
Ethnicity, no. (%) ^b			
White	74 (77.9)	150 (79.8)	0.983
Hispanic	12 (12.6)	22 (11.7)	
Black	7 (7.4)	12 (6.4)	
Other	2 (2.1)	4 (2.1)	
Smoking status, no. (%) ^b			
Never smoker	52 (54.7)	86 (45.7)	0.168
Former smoker	34 (35.8)	70 (37.2)	
Current smoker	9 (9.5)	32 (17.0)	
Pack-years, median (range)	25.0 (0.5–82.5)	17.6 (0.2–130.0)	0.283
Cigarettes/day, median (range)	20.0 (4.0–50.0)	20.0 (1.0–80.0)	0.339
Years smoking cigarettes, median (range)	25.0 (2.0–50.0)	22.5 (1.0–62.0)	0.498
BMI (kg/m ²), no. (%) ^b			
<25	17 (17.9)	56 (29.8)	0.007
25 to <30	30 (31.6)	72 (38.3)	
At least 30	48 (50.5)	60 (31.9)	
History of high blood pressure, no. (%) ^b			
Yes	54 (56.8)	75 (39.9)	0.007
No	41 (43.2)	113 (60.1)	

^a P -value for Student's t -test (continuous variables) or χ^2 test (categorical variables).

^bPercentages might not sum to 100% because of rounding.

Table II. Baseline and NNKOAc-induced DNA damage in renal cancer patients and control subjects

Treatment	Case patients		Control subjects		P -value ^a	P -value ^b
	No.	Olive tail moment, median (range)	No.	Olive tail moment, median (range)		
Baseline ^c	90	1.08 (0.44–3.77)	175	1.02 (0.45–2.85)	0.112	0.943
NNKOAc ^d	95	2.27 (0.61–7.78)	188	1.76 (0.48–5.77)	0.002	0.040
NNKOAc–baseline ^e	90	1.02 (–1.47–4.84)	175	0.78 (–0.85–4.64)	0.018	0.072

^a P -value for Wilcoxon rank sum test.

^b P -value for Wilcoxon rank sum test using a log-transformed Olive tail moment.

^cBaseline denotes DNA damage before NNKOAc.

^dNNKOAc denotes 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone-induced DNA damage.

^eNNKOAc–baseline denotes the net effect or baseline DNA damage subtracted from NNKOAc-induced DNA damage.

Table III. Baseline and NNKOAc-induced DNA damage in RCC patients and control subjects by smoking status

Treatment	Case		Control		<i>P</i> -value ^a	<i>P</i> -value ^b
	No.	Olive tail moment, median (range)	No.	Olive tail moment, median (range)		
Never smoker						
Baseline ^c	49	1.08 (0.45–2.95)	81	1.02 (0.49–2.36)	0.099	0.990
NNKOAc ^d	52	2.18 (0.61–7.78)	86	1.79 (0.74–5.67)	0.058	0.421
NNKOAc–baseline ^e	49	0.95 (–1.48–4.84)	81	0.74 (–0.48–4.64)	0.097	0.178
Former smoker						
Baseline ^c	33	1.12 (0.44–3.77)	66	1.06 (0.50–2.85)	0.876	0.767
NNKOAc ^d	34	2.42 (0.73–6.25)	70	1.96 (0.48–4.44)	0.023	0.021
NNKOAc–baseline ^e	33	1.19 (–0.03–4.43)	66	0.85 (–0.52–2.75)	0.038	0.092
Current smoker						
Baseline ^c	8	1.05 (0.70–2.80)	28	0.94 (0.45–2.47)	0.128	0.790
NNKOAc ^d	9	1.89 (0.99–4.72)	32	1.58 (0.58–5.77)	0.345	0.950
NNKOAc–baseline ^e	8	0.53 (–0.41–3.67)	28	0.70 (–0.85–3.29)	0.761	0.704

^a*P*-value for Wilcoxon rank sum test.^b*P*-value for Wilcoxon rank sum test using a log-transformed Olive tail moment.^cBaseline denotes DNA damage before NNKOAc.^dNNKOAc denotes 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone-induced DNA damage.^eNNKOAc–baseline denotes the net effect or baseline DNA damage subtracted from NNKOAc-induced DNA damage.**Table IV.** Estimates of RCC for baseline and NNKOAc-induced DNA damage quantified by the olive tail moment parameter

Analysis method	Case patients	Control subjects	Multivariate OR (95% CI) ^a
Baseline ^b			
By 75th percentile value, no. (%) ^c			
Low (<1.342)	65 (72.2)	131 (74.9)	Reference
High (≥1.342)	25 (27.8)	44 (25.1)	1.07 (0.59–1.97)
By quartiles, no. (%) ^c			
First (≤0.788)	19 (21.1)	44 (25.1)	Reference
Second (0.789–1.017)	14 (15.6)	44 (25.1)	0.71 (0.30–1.68)
Third (1.018–1.342)	32 (35.6)	43 (24.6)	1.62 (0.76–3.44)
Fourth (>1.342)	25 (27.8)	44 (25.1)	1.21 (0.56–2.62)
<i>P</i> for trend			0.298
NNKOAc ^d			
By 75th percentile value, no. (%) ^c			
Low (<2.478)	56 (59.0)	141 (75.0)	Reference
High (≥2.478)	39 (41.1)	47 (25.0)	2.06 (1.17–3.61)
By quartiles, no. (%) ^c			
First (≤1.183)	13 (13.7)	47 (25.0)	Reference
Second (1.184–1.762)	20 (21.1)	47 (25.0)	1.61 (0.69–3.75)
Third (1.763–2.478)	23 (24.2)	47 (25.0)	1.77 (0.76–4.09)
Fourth (≥2.479)	39 (41.1)	47 (25.0)	3.00 (1.36–6.62)
<i>P</i> for trend			0.006
NNKOAc–baseline ^e			
By 75th percentile value, no. (%) ^c			
Low (<1.350)	54 (60.0)	131 (74.9)	Reference
High (≥1.350)	36 (40.0)	44 (25.1)	1.90 (1.06–3.40)
By quartiles, no. (%) ^c			
First (≤0.300)	16 (17.8)	42 (24.0)	Reference
Second (0.301–0.775)	18 (20.0)	46 (26.3)	1.13 (0.49–2.62)
Third (0.776–1.350)	20 (22.2)	43 (24.6)	1.52 (0.66–3.49)
Fourth (≥1.351)	36 (40.0)	44 (25.1)	2.29 (1.06–5.00)
<i>P</i> for trend			0.021

^aAdjusted for age (±5 years), sex, ethnicity, smoking status, BMI and history of high blood pressure.^bBaseline denotes DNA damage before NNKOAc.^c75th percentiles and quartiles determined by the distribution of the controls.^dNNKOAc denotes 4-[(acetoxymethyl) nitrosamino]-1-(3-pyridyl)-1-butanone-induced DNA damage.^eNNKOAc–baseline denotes the net effect or baseline DNA damage subtracted from NNKOAc-induced DNA damage.

associated with a significantly increased risk of RCC. Furthermore, in quartile analysis, there was a dose–response association between NNKOAc-induced damage and risk of RCC.

Inter-individual differences in DNA repair capacity have been suggested to impact cancer susceptibility in the general population. Mutagen-induced DNA damage level reflects a constitutive DNA repair capacity in response to the mutagen challenge. Our data showed that individuals with higher sensitivity to NNKOAc-induced DNA damage exhibited a higher risk for RCC. It has been well established that genome stability is maintained by the host's DNA repair system (9). Defects in the DNA repair system leads to sensitivity to environmental exposures, accumulation of mutations and increased risk of cancer development (21,22). Hsu hypothesized that genetic susceptibility to mutagen damage varies along a continuum in the general population (23), with recognized repair deficiency syndromes such as Fanconi's anemia and ataxia telangiectasia located at the most extreme of the continuum. In response to insults by environmental carcinogens, genetic damage accumulates more rapidly in individuals with suboptimal DNA repair capacity than in those with normal repair capacity. Thus, people deficient in DNA repair capacity may have a higher risk for developing cancer. Therefore, quantification of individuals' susceptibility to DNA damage by *in vitro* mutagen challenge assays may facilitate identifying subgroups at increased risk of developing cancer.

The molecular basis of NNKOAc sensitivity associated with an increased risk for RCC is unclear. Studies have shown that NNK adducts mediate mutation of the tumor suppressor gene *k-Ras*, upregulate expression of c-Myc, Bcl-2 and cyclin-D1, activate adrenergic receptor signaling and increase DNA synthesis (24–29). *In vivo* studies have also shown that NNK exposure could induce chromosome instability by causing losses or gains in specific chromosomal regions; for example, losses on chromosomes 11 and 14 and gains on chromosomes 6 and 8 (30). However, the majority of these studies investigated the molecular mechanisms in lung tissue. Future studies focusing on molecular events occurring in renal tissue will be valuable to further our understanding of the molecular processes induced by NNK exposure, repair of NNK-induced DNA damage and RCC risk.

A limitation of current study is the small sample size that limits the statistical power to perform further stratified analysis. Future studies with larger sample sizes are warranted to investigate the association between NNKOAc sensitivity and RCC risk as modified by other risk factors such as cigarette smoking and obesity. However, as the first study of this kind, our data suggest that repair of the NNKOAc-induced DNA damage, as quantified by the comet assay, could be used as a prediction marker for RCC risk. Because DNA damage was measured post-cancer diagnosis, reverse causation, in which cancer disease status could lead to increased mutagen sensitivity, is a limitation of retrospective case–control studies. However, previous studies provided strong support to high genetic heritability of mutagen sensitivity (31–33) and

in a recent genotype–phenotype correlation study, Lin *et al.* (34) observed that sensitivity to benzo[*a*]pyrene diol epoxide-induced DNA damage was highly determined by genetic variants in the DNA repair pathway, further strengthening the argument that mutagen sensitivity is a genetic susceptibility marker but not a tumor marker.

In order to adjust for the possibility of laboratory drift over the one and a half year study period, we repeated all our analyses using a transformation of the Olive tail moment variables. We reported the *P*-values using the transformed data as a separate table column and did not observe major differences between the results of the original analyses and the results of the transformed analyses (Tables II–IV). Median NNKOAc-induced DNA damage remained significantly different between cases (0.41) and controls (0.29; *P*-value <0.05) and when baseline DNA damage was subtracted from NNKOAc-induced DNA damage, the results were borderline significantly different (*P*-value = 0.072). Likewise, among former smokers, cases remained to have significantly higher levels of NNKOAc-induced DNA damage as compared with controls (0.57 versus 0.28, *P* = 0.021). Furthermore, our logistic regression analyses remained consistent. For example, using the 75th percentile Olive tail moments of the controls as the cutoff point, we still observed higher levels of NNKOAc-induced DNA damage associated with a significantly increased risk of RCC (OR, 2.21; 95% CI, 1.25–3.88) and a significant dose–response association between NNK-induced damage (quartiles) and risk of RCC (*P* for trend, 0.036).

In the current population, we did not observe a significant association between cigarette smoking and RCC risk. However, it may be that this lack of association is due to variation in genetic susceptibility to DNA damage measured by the DNA damage/repair assay being independent of smoking. Therefore, in this study, we hypothesized that increased NNKOAc sensitivity, as measured by the comet assay, which partly reflects the host sensitivity to NNK in the cigarette smoking, predisposes individuals to increased risk of RCC.

A major strength of the current study is the possible insight our results might give to the lack of consistency in terms of RCC and cigarette smoking in the reported literature. It may be that this lack of association between cigarette smoking and RCC in our current population is due to variation in genetic susceptibility to DNA damage. Our results support this concept, in that we showed that genetic susceptibility to DNA damage caused by NNK, a major tobacco mutagen, is associated with an increased risk of RCC.

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References

1. Sasco, A.J. *et al.* (2004) Tobacco smoking and cancer: a brief review of recent epidemiological evidence. *Lung Cancer*, **45** (suppl. 2), S3–S9.
2. Hunt, J.D. *et al.* (2005) Renal cell carcinoma in relation to cigarette smoking: meta-analysis of 24 studies. *Int. J. Cancer*, **114**, 101–108.
3. Dhote, R. *et al.* (2000) Risk factors for adult renal cell carcinoma: a systematic review and implications for prevention. *BJU Int.*, **86**, 20–27.
4. Hung, R.J. *et al.* (2007) Family history and the risk of kidney cancer: a multicenter case-control study in Central Europe. *Cancer Epidemiol. Biomarkers Prev.*, **16**, 1287–1290.
5. Hecht, S.S. *et al.* (1988) Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis*, **9**, 875–884.
6. Wogan, G.N. *et al.* (2004) Environmental and chemical carcinogenesis. *Semin. Cancer Biol.*, **14**, 473–486.
7. Hoffmann, D. *et al.* (1982) Tobacco specific N-nitrosamines: occurrence and bioassays. *IARC Sci. Publ.*, **41**, 309–318.
8. Hecht, S.S. (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem. Res. Toxicol.*, **11**, 559–603.
9. Wood, R.D. *et al.* (2001) Human DNA repair genes. *Science*, **291**, 1284–1289.
10. Vineis, P. (2004) Individual susceptibility to carcinogens. *Oncogene*, **23**, 6477–6483.
11. Berwick, M. *et al.* (2000) Markers of DNA repair and susceptibility to cancer in humans: an epidemiologic review. *J. Natl Cancer Inst.*, **92**, 874–897.
12. Brown, P.J. *et al.* (2008) Repair of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced DNA pyridyloxobutylation by nucleotide excision repair. *Cancer Lett.*, **260**, 48–55.
13. Lin, X. *et al.* (2007) Risk assessment of renal cell carcinoma using alkaline comet assay. *Cancer*, **110**, 282–288.
14. Wu, X. *et al.* (2004) Genetic susceptibility to tobacco-related cancer. *Oncogene*, **23**, 6500–6523.
15. Olive, P.L. *et al.* (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the “comet” assay. *Radiat. Res.*, **122**, 86–94.
16. Singh, N.P. *et al.* (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.
17. Cloutier, J.F. *et al.* (2001) Characterization and mapping of DNA damage induced by reactive metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) at nucleotide resolution in human genomic DNA. *J. Mol. Biol.*, **313**, 539–557.
18. Lacoste, S. *et al.* (2006) Formamidopyrimidine adducts are detected using the comet assay in human cells treated with reactive metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Mutat. Res.*, **600**, 138–149.
19. Lacoste, S. *et al.* (2007) Repair kinetics of specific types of nitroso-induced DNA damage using the comet assay in human cells. *Mutat Res.*, **624**, 18–30.
20. Wolz, L. *et al.* (2002) The comet assay with MCL-5 cells as an indicator of genotoxic treatment with chemicals and cigarette smoke condensates. *Altern. Lab. Anim.*, **30**, 331–339.
21. Schwartz, A.G. *et al.* (2007) The molecular epidemiology of lung cancer. *Carcinogenesis*, **28**, 507–518.
22. Spitz, M.R. *et al.* (2003) Genetic susceptibility to lung cancer: the role of DNA damage and repair. *Cancer Epidemiol. Biomarkers Prev.*, **12**, 689–698.
23. Hsu, T.C. (1983) Genetic instability in the human population: a working hypothesis. *Hereditas*, **98**, 1–9.
24. Belinsky, S.A. *et al.* (1989) Relationship between the formation of promutagenic adducts and the activation of the K-ras protooncogene in lung tumors from A/J mice treated with nitrosamines. *Cancer Res.*, **49**, 5305–5311.
25. Laag, E. *et al.* (2006) NNK activates ERK1/2 and CREB/ATF-1 via beta-1-AR and EGFR signaling in human lung adenocarcinoma and small airway epithelial cells. *Int. J. Cancer*, **119**, 1547–1552.
26. Ehrhardt, A. *et al.* (2003) Increased susceptibility to the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in transgenic mice overexpressing c-myc and epidermal growth factor in alveolar type II cells. *J. Cancer Res. Clin. Oncol.*, **129**, 71–75.
27. Ho, Y.S. *et al.* (2005) Tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces cell proliferation in normal human bronchial epithelial cells through NFκB activation and cyclin D1 up-regulation. *Toxicol. Appl. Pharmacol.*, **205**, 133–148.
28. Oreffo, V.I. *et al.* (1992) Mutational analysis of a dominant oncogene (c-Ki-ras-2) and a tumor suppressor gene (p53) in hamster lung tumorigenesis. *Mol. Carcinog.*, **6**, 199–202.
29. Jin, Z. *et al.* (2004) Tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone promotes functional cooperation of Bcl2 and c-Myc through phosphorylation in regulating cell survival and proliferation. *J. Biol. Chem.*, **279**, 40209–40219.
30. Herzog, C.R. *et al.* (2006) Array CGH analysis reveals chromosomal aberrations in mouse lung adenocarcinomas induced by the human lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Biochem. Biophys. Res. Commun.*, **341**, 856–863.
31. Cloos, J. *et al.* (1994) Increased mutagen sensitivity in head-and-neck squamous-cell carcinoma patients, particularly those with multiple primary tumors. *Int. J. Cancer*, **56**, 816–819.
32. Roberts, S.A. *et al.* (1999) Heritability of cellular radiosensitivity: a marker of low-penetrance predisposition genes in breast cancer? *Am. J. Hum. Genet.*, **65**, 784–794.
33. Wu, X. *et al.* (2006) Mutagen sensitivity has high heritability: evidence from a twin study. *Cancer Res.*, **66**, 5993–5996.
34. Lin, J. *et al.* (2007) Mutagen sensitivity and genetic variants in nucleotide excision repair pathway: genotype-phenotype correlation. *Cancer Epidemiol. Biomarkers Prev.*, **16**, 2065–2071.

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